






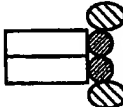
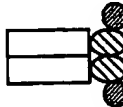




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁶ : C07K 19/00, A61K 47/48 // 38/20, C07K 14/54, 16/30</p>	A2	<p>(11) International Publication Number: WO 99/29732</p> <p>(43) International Publication Date: 17 June 1999 (17.06.99)</p>
<p>(21) International Application Number: PCT/US98/25978</p> <p>(22) International Filing Date: 8 December 1998 (08.12.98)</p> <p>(30) Priority Data: 08/986,997 8 December 1997 (08.12.97) US</p> <p>(71) Applicant: LEXIGEN PHARMACEUTICALS CORPORATION [US/US]; 125 Hartwell Avenue, Lexington, MA 02173 (US).</p> <p>(72) Inventors: GILLIES, Stephen, D.; 159 Sunset Road, Carlisle, MA 01741 (US). LO, Kin-Ming; 6 Carol Lane, Lexington, MA 02173 (US). LAN, Yan; 21 Newton Street, Belmont, MA 02178 (US).</p> <p>(74) Agent: WALLER, Patrick, R., H.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).</p>		
<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>		
<p>(54) Title: HETERODIMERIC FUSION PROTEINS USEFUL FOR TARGETED IMMUNE THERAPY AND GENERAL IMMUNE STIMULATION</p>		
<p>(57) Abstract</p> <p>Disclosed are methods for producing fusion proteins with the heterodimeric cytokine, interleukin-12. In order to insure that the proper ration of fused and non-fused subunits are obtained in the fusion protein, a specific stepwise approach to genetic engineering is used. This consists of first expressing the non-fused p40 IL-12 subunit in a production cell line, followed by or simultaneously expressing in the same cell, a second recombinant fusion protein consisting of the fused polypeptide linked by a peptide bond to the p35 subunit of IL-12. Molecules containing the p35 fusion protein cannot be secreted from the transfected mammalian cell without first complexing in a one to ratio with the p40 subunit, thus ensuring the production of active heterodimeric fusion proteins.</p>		
<div style="display: flex; justify-content: space-around; align-items: flex-start;"> <div style="text-align: center;">  <p>p35</p> </div> <div style="text-align: center;">  <p>p40</p> </div> </div> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 10px;"> <div style="text-align: center;">  <p>Fc</p> </div> <div style="text-align: center;">  <p>Fc-dimer</p> </div> </div> <div style="margin-top: 20px;"> <div style="display: flex; align-items: center; margin-bottom: 10px;">  <div style="margin-left: 10px;">Fc-p35(NOT SECRETED)</div> </div> <div style="display: flex; align-items: center; margin-bottom: 10px;">  <div style="margin-left: 10px;">Fc-p40 (SECRETED)</div> </div> <div style="display: flex; align-items: center; margin-bottom: 10px;">  <div style="margin-left: 10px;">Fc-p35/Fc-p40 (SECRETED)</div> </div> <div style="display: flex; align-items: center; margin-bottom: 10px;">  <div style="margin-left: 10px;">Fc-p35/p40 (SECRETED)</div> </div> <div style="display: flex; align-items: center;">  <div style="margin-left: 10px;">Fc-p40/p35 (SECRETED)</div> </div> </div>		

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HETERODIMERIC FUSION PROTEINS USEFUL FOR TARGETED IMMUNE THERAPY AND GENERAL IMMUNE STIMULATION

Field of the Invention

The present invention relates generally to fusion proteins. More specifically, the present invention relates to heterodimeric fusion proteins useful for targeted immune therapy and general immune stimulation.

5

Background of the Invention

One of the key immune regulators is the T helper cell which reacts to antigens presented on HLA class II molecules. This CD4⁺ cell differentiates in response to antigenic stimulation and becomes a type 1 or type 2 helper (Th1 or Th2) according to the type of cytokines that it secretes. Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173 (1989). A Th1 response leads to the
10 secretion of interleukin-2 (IL-2) and interferon- γ (IFN- γ) which stimulates cell-mediated immune reactions against intracellular pathogens. A Th2 response leads to the secretion of IL-4, IL-5 and IL-10 which stimulates antibody responses to extracellular pathogens. The most interesting component of this system of regulation is that one response inhibits the other through the negative regulatory activities of the cytokines that are produced. Thus, IL-4 and IL-10 can down-regulate
15 Th1 responses while IFN- γ can down-regulate Th2 responses.

The regulatory activity of T helper cells and their differentiation following exposure to antigen is regulated by cytokines as well. IL-12, a disulfide-linked heterodimeric cytokine with a 40 kDa subunit and 35 kDa subunit, exerts a powerful positive regulatory influence on the development of Th1 helper T-cell immune responses. See review by Trinchieri, *Blood* 84:
20 4008-4027 (1994). IL-12 also has a powerful synergistic effect in the induction of IFN- γ from both T helpers and natural killer (NK) cells (Eur. Patent Appl. 90123670.3). Secreted IFN- γ then inhibits any Th2 cell proliferation and polarizes the response to favor cell-mediated immunity.

One way of changing the outcome of an immune response would be to administer the appropriate cytokine at the time of antigen stimulation. If IL-4 was the major cytokine present
25 during antigen stimulation, the Th2 response would be enhanced and the Th1 response would be inhibited. In contrast, if IL-12 was the major cytokine present during antigen stimulation, the Th1

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response would be enhanced and the Th2 response would be inhibited. However, systemic administration of cytokines is difficult due to their very short circulating half-lives and their deleterious side effects.

A better approach is to target the effect of the cytokine to a cell surface antigen by fusing
5 it to an antibody (or fragment derived therefrom) having specificity and affinity for that antigen. See Gillies, *et al.*, *Proc. Natl. Acad. Sci.* 89: 1428-1432 (1992); U.S. Patent No 5,650,150, the disclosure of which is incorporated herein by reference. Alternatively, the stimulatory cytokine can be linked to a protein antigen via a peptide linkage in the form of a fusion protein. See
10 Hazama, *et al.*, *Vaccine* 11: 629-636 (1993). However, the complex structure of IL-12 makes it more difficult to express as a fusion protein due to the necessity of expressing exactly the same molar ratio of each subunit in the final product. In fact, IL-12 itself is naturally expressed and secreted as a mixture of p40 homodimer. D'Andrea, *et al.*, *J. Exp. Med.*, 176: 1387-1398
(1992).

Therefore, there is a need in the art for methods of producing fusion proteins with
15 heterodimeric cytokines and an antibody or an antigen that maintain the natural heterodimeric structure of the cytokine and secretes the molecules with equimolar ratios of the subunits.

Summary of the Invention

The present invention provides heterodimeric fusion proteins useful for targeted immune therapy and general immune stimulation and methods for producing these heterodimeric fusion
20 proteins. Specifically, the present invention provides methods for the production of fusion proteins with IL-12 that maintain its natural heterodimeric structure, and provide for the secretion of the molecules with equimolar ratios of IL-12 subunits.

In one aspect of the invention, the fusion proteins comprise a heterodimeric cytokine linked to an antibody, or a portion thereof. In a preferred embodiment, the fusion protein
25 comprises two chimeric chains linked by a disulfide bond. Each chimeric chain comprises a different subunit of the heterodimeric cytokine linked through a peptide bond to a portion of an Ig heavy chain.

In an alternative preferred embodiment, the fusion protein comprises a first chimeric chain comprising one of the subunits of the heterodimeric cytokine linked by a peptide bond to a

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portion of an Ig heavy chain. This subunit is linked by a disulfide bond to the other subunit of the heterodimeric cytokine. In another alternative preferred embodiment, this first chimeric chain is linked by a disulfide bond to a second chimeric chain comprising one of the subunits of the heterodimeric cytokine linked by a peptide bond to a portion of an Ig heavy chain and by a
5 disulfide bond to the other subunit of the heterodimeric cytokine.

In yet another alternative preferred embodiment, the fusion protein is a trimeric fusion protein comprising a first and a second chimeric chain linked by a disulfide bond. Each chimeric chain comprises a subunit of the heterodimeric cytokine linked by a peptide bond to a portion of an Ig heavy chain. The subunit of one of the chimeric chains is further linked by a disulfide bond
10 to a different subunit of the heterodimeric cytokine.

Fusion proteins of the invention may be considered chimeric by virtue of two aspects of their structure. First, the fusion protein is chimeric in that it includes an immunoglobulin chain (typically but not exclusively a heavy chain) of appropriate antigen-binding specificity fused to a given heterodimeric cytokine. Second, an immunoconjugate of the invention may be chimeric in
15 the sense that it includes a variable region and a constant region which may be the constant region normally associated with the variable region, or a different one and thus a V/C chimera; *e.g.*, variable and constant regions from different species. Also embraced within the term "fusion protein" are constructs having a binding domain comprising framework regions and variable regions (*i.e.*, complementarity determining regions) from different species, such as are disclosed
20 by Winter, *et al.*, GB2, 188, 638.

The heterodimeric cytokine-antibody fusion protein of the present invention preferably displays antigen-binding specificity. In a preferred embodiment, the heterodimeric cytokine-antibody fusion protein comprises a heavy chain. The heavy chain can include a CH1, CH2, and/or CH3 domains. In an alternative preferred embodiment, the heterodimeric cytokine-
25 antibody fusion protein comprises a light chain. The invention thus provides fusion proteins in which the antigen binding specificity and activity of an antibody are combined with the potent biological activity of a heterodimeric cytokine. A fusion protein of the present invention can be used to deliver selectively a heterodimeric cytokine to a target cell *in vivo* so that the heterodimeric cytokine can exert a localized biological effect.

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Preferably, the fusion protein of the present invention displays cytokine biological activity. The preferred heterodimeric cytokine of the fusion protein is IL-12. Fusions with antibodies capable of binding antigens are useful for co-localizing the immune stimulatory activity of IL-12 either to target cells or target protein antigens.

5 Further, the fusion protein of the present invention preferably has a longer circulating half-life than an unlinked heterodimeric cytokine. Fusions with the Fc portion of antibodies and IL-12 are useful for altering the pharmacology and biodistribution of the molecule by increasing its circulating half-life and its affinity for Fc-receptor bearing cells, *e.g.*, antigen presenting cells. Changes in biodistribution may also alter its systemic toxicity by changing the mechanism by
10 which it is cleared from the circulation.

In another aspect of the invention, the fusion proteins comprise a heterodimeric cytokine linked to an antigen. The preferred heterodimeric cytokine-antigen fusion protein of the present invention displays cytokine biological activity and antigenic activity. Further, the fusion protein of the present invention preferably has a longer circulating half-life than an unlinked heterodimeric
15 cytokine. The preferred heterodimeric cytokine of the fusion protein is IL-12.

In a preferred embodiment, the fusion protein comprises two chimeric chains linked by a disulfide bond. Each chimeric chain comprises a different subunit of the heterodimeric cytokine, either of which is linked through a peptide bond to an antigen.

In an alternative preferred embodiment, the fusion protein comprises a first chimeric chain
20 comprising one of the subunits of the heterodimeric cytokine linked by a peptide bond to an antigen. This subunit is linked by a disulfide bond to the other subunit of the heterodimeric cytokine. In another alternative preferred embodiment, this first chimeric chain is linked by a disulfide bond to a second chimeric chain comprising one of the subunits of the heterodimeric cytokine linked by a peptide bond to an antigen and by a disulfide bond to the other subunit of the
25 heterodimeric cytokine.

In another alternative preferred embodiment, the fusion protein is a trimeric fusion protein comprising a first and a second chimeric chain linked by a disulfide bond. Each chimeric chain comprises a subunit of the heterodimeric cytokine linked by a peptide bond to an antigen. The

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subunit of one of the chimeric chain is further linked by a disulfide bond to a different subunit of the heterodimeric cytokine.

The invention also features DNA constructs encoding the above-described fusion proteins, and cell lines, *e.g.*, myelomas, transfected with these constructs.

5 The invention also includes a method for selectively targeting a heterodimeric cytokine. In a preferred embodiment, the method comprise linking at least one subunit of a heterodimeric cytokine by a peptide bond to a portion of an Ig heavy chain. In an alternative preferred embodiment, the method comprise linking each of the two subunits of a heterodimeric cytokine by a peptide bond to a portion of an Ig heavy chain, thereby forming two chimeric chain. The two
10 chimeric chains are linked by a disulfide bond, thereby forming a heterodimeric fusion protein. In yet another preferred embodiment, the method comprises (1) linking one of the two subunits of a first heterodimeric cytokine by a peptide bond to an Ig heavy chain, thereby forming a first chimeric chain; (2) linking one of the two subunits of a second heterodimeric cytokine by a peptide bond to an Ig heavy chain, thereby forming a second chimeric chain; and (3) linking the
15 first and second chimeric chains by a disulfide bond, thereby forming a fusion protein. The resulting fusion proteins can display binding specificity for a predetermined antigen and cytokine biological activity.

 The invention also includes a method of selectively delivering a heterodimeric cytokine to a target cell. The method includes providing a heterodimeric cytokine fusion protein including a
20 chimeric Ig chain including an Ig heavy chain having a variable region specific for an epitope on the target cell and a constant region joined at its carboxy terminus by a peptide bond to a cytokine, and an Ig light chain combined with the chimeric Ig heavy chain, forming a functional antigen-binding site, and administering the fusion protein in an amount sufficient to reach the target cell to a subject harboring the target cell.

25 Further, the invention features a method of increasing the circulating half-life of a heterodimeric cytokine. In a preferred embodiment, the method comprise linking at least one subunit of a heterodimeric cytokine by a peptide bond to a polypeptide. In an alternative preferred embodiment, the method comprises linking each of the two subunits of a heterodimeric cytokine by a peptide bond to a polypeptide, thereby forming two chimeric chain. The two
30 chimeric chains are linked by a disulfide bond, thereby forming a heterodimeric fusion protein. In

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yet another preferred embodiment, the method comprises (1) linking one of the two subunits of a first heterodimeric cytokine by a peptide bond to a polypeptide, thereby forming a first chimeric chain; (2) linking one of the two subunits of a second heterodimeric cytokine by a peptide bond to a polypeptide, thereby forming a second chimeric chain; and (3) linking the first and second
5 chimeric by a disulfide bond, thereby forming a fusion protein. The polypeptide can be serum albumin, an antigen, and a portion of an Ig heavy chain. The resulting fusion proteins display cytokine biological activity.

The IL-12 fusion proteins of the present invention are useful for specific targeting or immune stimulation when it is important to generate a cell-mediated immune response, such as in
10 cancer immunotherapy or antiviral responses. They are also useful for specifically downregulating Th2 responses which often lead to the overproduction of IL-4. This cytokine has been shown to be essential for the development of allergy through the induction of a Th2 response and the resulting overproduction of IgE antibody.

Brief Description of the Drawings

15 The foregoing and other objects of the present invention, and the various features thereof, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIG. 1 is a diagrammatic representation of the predicted protein structure of heterodimeric fusion proteins;

20 FIG. 2 is a diagrammatic representation of an SDS-PAGE showing an analysis, under reducing conditions, of proteins secreted by cells transfected with vectors expressing the Fc-p35 fusion protein (lane 1), the Fc-p40 fusion protein (lane 2), the Fc-p35 fusion protein and the Fc-p40 fusion protein (lane 3), the Fc-p35 fusion protein and the p40 subunit (lane 4), and the p35 subunit and the Fc-p40 fusion protein (lane 5);

25 FIG. 3 is a diagrammatic representation of the predicted protein structure of expressed fusion proteins;

FIG. 4 is a bar graph depicting the ability of various fusion proteins to stimulate IFN- γ production;

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FIG. 5A is a diagrammatic representation of an SDS-PAGE showing an analysis of whole antibody-IL-12 fusion proteins produced by two independent transfectants, under non-reducing (lanes 1 and 2) and reducing conditions (lanes 3 and 4);

FIG. 5B-D are line graphs depicting the effects of human IL-12 (X), Hu-KS-IL-12 fusion protein with both human IL-12 chains (closed squares), and Hu-KS-1/4-mouse p35 human p40 fusion protein (open squares) on proliferation of mitogen-activated human PBMC (Panel B); induction of IFN- γ secretion from PHA-activated PBMC (Panel C) and from mouse effector cells, pre-stimulated with Concanavalin A (Panel D);

FIG. 6A-B are line graphs depicting the effects of IL-12 (X), single-chain fusion protein with human p35 and p40 subunits (closed squares), and single-chain fusion protein with a mouse p35 subunit and a human p40 subunit (open squares) on induction of IFN- γ secretion;

FIG. 6C is line graphs depicting the antigen binding activity of whole Hu-KS-1/4-IL-12 fusion protein (open squares), single-chain fusion protein with human IL-12 (open diamond), single-chain fusion protein with mouse p35 human p40 (open and free circles), and human IL-12 (open triangles);

FIG. 7 is a graph depicting the serum half-life of Hu-KS-IL-12 (mouse p35 human p40), as measured by an ELISA using a capture step with anti-human H and L chain and a second detection with either anti-human Fc antibody (closed diamonds) or anti-human IL-12 p40 antibody (open squares);

FIG. 8 (top and bottom panels) are line graphs depicting the immunogenicity of IL-12 fusion proteins. Serum dilutions from animals injected with either Hu-KS-1/4 antibody or Hu-KS-1/4-IL-12 (mouse p353 human p40) were tested for reactivity to Hu-KS-1/4 antibody.

Detailed Description of the Invention

The present invention describes fusion proteins between heterodimeric cytokines and other proteins. Heterodimeric cytokines can be fused to, for example, proteins with targeting or antigenic properties. Fusion proteins between heterodimeric cytokines and proteins with targeting or antigenic properties may have a longer circulating half life than unlinked heterodimeric cytokines. Targeting or antigenic properties are not required for the increased circulating half life

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as this property can also be achieved by fusing a heterodimeric cytokine with a protein that lacks targeting or antigenic properties such as, for example, serum albumin.

The fusion proteins of this invention can be produced by genetic engineering techniques. As depicted in FIG. 1, various fusion protein constructs can be produced by the methods of the present invention. In one embodiment, one of the subunit of the heterodimeric cytokine fused to a polypeptide is co-expressed with a free subunit of the other type. Once expressed, the chimeric chain is linked by a disulfide bond to the free subunit (FIG. 1B). In another embodiment, the polypeptide fused with one of the subunit can be linked to another such polypeptide. Since each polypeptide is linked to a heterodimeric cytokine, the resulting construct has two molecules of the heterodimeric cytokine (FIG. 1C). In yet another embodiment, each of the subunit of the heterodimeric cytokine is fused to a polypeptide and the two chimeric chains are linked by a disulfide bond. The resulting construct has only one molecule of the heterodimeric cytokine (FIG. 1D). In yet another embodiment, two subunits of the heterodimeric cytokine fused to a polypeptide are co-expressed with a free subunit. The resulting construct has three subunits of the heterodimeric cytokine (FIG. 1E).

At present, the only known heterodimeric cytokine is IL-12. However, as novel heterodimeric cytokines are identified and sequenced, a skilled artisan will be able to use methods of the present invention to produce fusion proteins with these novel heterodimeric cytokines.

Methods for synthesizing useful embodiments of the invention are described, as well as assays useful for testing their pharmacological activities, both *in vitro* and in pre-clinical *in vivo* animal models. The preferred gene construct encoding a chimeric chain (*i.e.*, a subunit of the heterodimeric cytokine fused to a polypeptide) includes, in 5' to 3' orientation, a DNA segment which encodes a polypeptide and DNA coding for one subunit of the heterodimeric cytokine. An alternative preferred gene construct includes, in 5' to 3' orientation, a DNA segment which encodes one subunit of the heterodimeric cytokine and DNA coding for a polypeptide. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells where it is expressed.

The invention is illustrated further by the following non-limiting examples:

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Example 1 Cloning cDNAs encoding human and mouse IL-12 subunits

Human peripheral blood monocytes (PBMC) were obtained from a healthy volunteer and were purified by centrifugation on a Ficoll-Hypaque (Pharmacia) gradient (1700 rpm for 20 min). The "buffy" coat containing the PBMC was diluted with serum-free culture medium (SF-RPMI) to a volume of 50 ml and collected by centrifugation at 1500 rpm for 5 min. Cells were resuspended in AIM-V cell culture medium (GIBCO) at a density of 5×10^6 cells/ml and were cultured for 2 days at 37°C in a humidified CO₂ incubator. The attached cells were selected by gently agitating the culture flask to remove non-adherent cells. Fresh medium containing phorbol ester (100 nM) and the calcium ionophore, ionomycin (0.1 µg/ml) was added. After three days, the cells were collected by gentle scraping and centrifugation. Poly A+ mRNA was prepared using oligo dT-coated beads (Dynal, Inc.).

Subunit cDNAs were cloned using polymerase chain reactions (PCR). First strand cDNA was synthesized in a 50 µl reaction containing oligo dT primer (50 µg/ml), reaction buffer, RNasin (10 U/ml) and reverse transcriptase. Incubation was at 43°C for 2 hrs, followed by extraction with phenol, phenol:chloroform (50:50) and precipitation with ethanol. The cDNA product was used as template for PCR reactions containing Taq polymerase and reaction buffer (10x buffer; Perkin Elmer), sense and antisense primers (0.2 to 0.5 µM each), and 10% of the cDNA reaction. Primer sequences were 5'-CCAGAAAGCAAGAGACCAGAG-3' (SEQ ID NO: 1) for the sense primer, and 5'-GGAGGGACCTCGAGTTTTAGGAAGCATTTCAG-3' (SEQ ID NO: 2) for the antisense primer of the p35 subunit cDNA. The sense primer is derived from a sequence in the 5' untranslated region of the p35 message just upstream of a XmaI site, while the antisense primer encodes a translational stop codon followed shortly thereafter by a convenient XhoI site for directional subcloning in an expression vector. The primers for the p40 subunit cDNA were 5'-CTCCGTCCTGTCTAGAGCAAGATGTGTC-3' (SEQ ID NO: 3) for the sense and 5'-GCTTCTCGAGAACCTAACTGCAGGGCACAG-3' (SEQ ID NO: 4) for the antisense primer. The sense primer encodes a unique XbaI site upstream of the translation start site while the antisense primer encodes a stop codon and unique XhoI site as above. Both subunit sequences, cloned with these PCR primers, will be expressed as single proteins and thus require native (or other) secretory leader sequences for proper heterodimer assembly and secretion. PCR reactions consisted of 40 cycles including: 1 min at 92°C, 2 min at 52°C, and 3 min at 72°C,

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following an initial denaturation step at 94°C for 2 min. Products were gel purified and cloned in the SK cloning vector (Stratagene) for sequence verification. DNA sequencing using a commercial kit (U.S. Biochemical) was carried out on each of the subunit cDNA. The same procedure can be used to clone the mouse p35 subunit cDNA from spleen cells activated with

5 Concanavalin A (5 µg/ml in culture medium for 3 days). Recommended primers are 5'-CCTCTACTAACATGTGTCAATCACGCTACCTC-3' (SEQ ID NO: 5) for the sense and 5'-CCCTCGAGTCAGGCGGAGCTCAGATAGCC-3' (SEQ ID NO: 6) for the antisense primers encoding the same restriction sites as described above for the human p35 subunit.

Example 2 Expression of fusion protein combinations in transfected mammalian cells

10 In order to make the fused versions of each subunit, the DNAs encoding the mature protein sequence of each were adapted as follows. The p40 subunit DNA was digested with NdeI which cuts very close to the junction of the mature protein and leader sequence, and XhoI. An adapter oligonucleotide was synthesized with the sequence 5'-CCGGGCAAGTCCA-3' (SEQ ID NO: 7) hybridized to a second, partly complementary oligonucleotide with the sequence

15 5'-TATGGACTTGC-3' (SEQ ID NO: 8). The double stranded DNA contains overhanging sequence compatible with ligation to an XmaI site at the 5' end and an NdeI site at the 3' end. This fragment was ligated to the NdeI-XhoI fragment of the p40 cDNA and cloned as an XmaI to XhoI fragment in vector pC-Fc-X, cut with XmaI and XhoI. This vector already contains a human IgG1 Fc encoding DNA fragment in its genomic configuration (containing introns and

20 exons) and fused downstream of a leader sequence derived from a mouse light chain. *See, Gillies, et al., J. Immunol. Methods 125: 191-202 (1989).* The addition of a DNA fragment to its unique XmaI site allows for the production of fusion proteins joined directly to the carboxyl terminus of the Fc, provided that the reading frame between the two sequences is maintained (Lo, *et al.*, U.S. Patent No. 5,541,087). Other proteins (*e.g.*, antigen, serum albumin) can be fused to the amino

25 termini of these subunits in the same manner. The advantages of this method include the large quantities of product produced and the ease of purification of the product by binding to and elution from protein A Sepharose.

The same general strategy was used to fuse the p35 subunit DNA to human Fc. In this case, a XmaI-BalI linker was synthesized using the oligonucleotides

30 5'-CCGGGAAGAAACCTCCCCGTGG-3' (SEQ ID NO: 9) and

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5'-CCACGGGGAGGTTTCTTC-3' (SEQ ID NO: 10), which were ligated to a p35 subunit DNA, cut with *BalI* and *XhoI*, and subcloned as an *XmaI*-*XhoI* fragment in the pdC-Fc-X vector, as described above. The human p35 subunit has been shown to be active for human cells but not mouse cells, in terms of IL-12 activity, whereas the human p40 subunit does not show species
5 specificity. Therefore, the human p40 subunit can be used to make either all human IL-12 fusion proteins or hybrid human/mouse fusion proteins.

The resulting constructs encode Fc-p35 or Fc-p40 fusion proteins which are expected to spontaneously dimerize into proteins of 120 kD (50 kD from the Fc) and 130 kD respectively and to migrate after reduction on denaturing SDS gels as proteins of 60 kD and 65 kD. The
10 individual subunit cDNAs were subcloned in the pdC expression vector (without the Fc) for their expression as independent proteins. This vector provides promoter sequences for expression of mRNA, transcribed from the cDNA insert, following the transfection of mammalian cells. It also provides for a 3' untranslated region and poly A addition site, downstream of the 3' *XhoI* insertion site. There are also sequences necessary for the propagation of the plasmid in *E. coli*
15 and selection with ampicillin, as well as a selectable marker gene, such as dihydrofolate reductase (*dhfr*), for conferring resistance to methotrexate. These same components are also used in the pdC-Fc-X vector for expression of the fusion proteins.

For expression of biologically-active IL-12 fusion protein heterodimers, different combinations of the individual vectors encoding fusion and non-fusion forms of the subunits were
20 transiently expressed by co-transfection of human 293 epidermal carcinoma cells. DNA was purified using preparative kits (Wizard, Promega Inc.), ethanol precipitated for sterilization and resuspension in sterile water. Calcium phosphate precipitates were prepared by standard methods using 10 µg of DNA per ml (5 µg of each when two plasmids were co-transfected) and 0.5 ml/plate were added to cultures of 293 growing in 60 mm plates at approximately 70%
25 confluency. MOLECULAR CLONING A LABORATORY MANUAL, 2nd Ed. (Sambrook, Fritsch and Maniatis, eds., Cold Spring Harbor Laboratory Press, 1989). After 16 hr, the medium containing the precipitate was removed and replaced with fresh medium. After 3 days, the supernatant was removed and analyzed for production of transfected gene expression by ELISA, biological determination of IL-12 activity, or immunoprecipitation and analysis on SDS gels of radioactively
30 labeled proteins. For labeling, medium without methionine was used to replace the growth

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medium on the second day of culture and ^{35}S -methionine (100 $\mu\text{Ci/ml}$) is added. After an additional 16 hr incubation, the media was harvested, clarified by centrifugation (5 min at 13,000 rpm in a table top microcentrifuge) and incubated with protein A Sepharose beads (10 μl of bead volume per ml of culture supernatant). After 1 hr at room temperature, the beads were washed by
5 repeated centrifugation and resuspension in PBS buffer containing 1% NP-40. The final pellet was resuspended in SDS-containing gel buffer and boiled for 2 min. After removing the beads by centrifugation, the supernatant was divided into two aliquots. Reducing agent (5% 2-mercaptoethanol) was added to one sample and both are boiled for 5 min prior to loading on an SDS polyacrylamide gel. After electrophoresis the gel was exposed to X-ray film
10 (autoradiography).

An example of an analysis of the co-expression of various fusion proteins and individually expressed proteins, under reducing conditions, is shown in FIG. 2. The results show that the p35 subunit cannot be secreted from the cell, even when expressed as a fusion protein with the Fc fragment (lane 1). The p40 subunit, on the other hand, was readily secreted when fused to Fc
15 (lane 2). The p35 subunit was secreted when it could pair with the p40 subunit, either as an Fc-p35 fusion pairing with an Fc-p40 fusion protein (lane 3), the Fc-p35 pairing with free p40 (lane 4), or free p35 pairing with the Fc-p40 fusion protein (lane 5). In all cases of expression of a free subunit, together with a fusion protein, the free subunit assembles with the other subunit and forms a covalent, disulfide bond. A diagram of these various combinations is shown in FIG. 1.
20 Note that the construct with each subunit fused to Fc and co-expressed in the same cell has one molecule of IL-12 per Fc (FIG. 1D), whereas the constructs with a single subunit fusion to Fc paired with a free subunit (of the other type) has two molecules of IL-12 per Fc (FIG. 1C). Expression in stably transfected cells is expected to be different from transient expression since the expression and secretion is independent of p35. Thus, overexpression of p40 is possible and
25 more advantageous to the cell since it can easily be exported. This could lead to an overabundance of Fc-p40 subunits relative to Fc-p35 and result in a mixture of heterodimer and p40 homodimer secretion from the cell. This would be inefficient and lead to purification problems. Expression of p35 is likely to have a growth disadvantage, since excess protein is likely degraded in the endoplasmic reticulum, unless it is effectively paired with the p40 subunit. Thus,
30 it is possible to take advantage of this situation to ensure the balanced secretion of only heterodimer fusion product, by expressing the p35 subunit as a fusion protein together with free

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p40 subunit. Only p35 fusion protein paired with an equimolar amount of p40 subunit can be secreted. Purification of this product on protein A results in a homogeneous preparation of heterodimer. A diagrammatic representation of the predicted protein structure of expressed fusion proteins is provided in FIG. 3.

5 **Example 3 Activity of Fusion Proteins on in an IFN- γ Induction Assay**

Biological activity was measured in an IFN- γ induction assay using mitogen-activated human PBMC, purified as described in Example 1. After gradient centrifugation, cells were resuspended in cell culture medium containing 10% fetal bovine serum (RPMI-10) and phytohemagglutinin (PHA; 10 μ g/ml) at a density of 5×10^6 cells/ml and were cultured for 3 days
10 at 37°C in a humidified CO₂ incubator. The PHA-activated cells were collected by centrifugation, washed three times with an equal volume of SF-RPMI and resuspended in fresh RPMI-10 (1×10^6 cells /ml). Aliquots (100 μ l) were dispensed into the wells of multiple 96-well plates to give a final cell number of 10^5 per well. Test samples from culture medium were serially diluted in fresh culture medium and added to wells of the 96-well plate. Stimulation medium (50 μ l/well)
15 containing 10% serum and IL-2 (25 U/ml) was added. Control wells received only IL-2 (negative control) or both IL-2 and commercial IL-12 (R & D Systems) but no sample (positive control). The plates were incubated for 48 hr at 37°C in a CO₂ incubator at which time aliquots (20 μ l) were removed for analysis of IFN- γ concentration by ELISA.

The same assay was used to determine the activity of mouse forms of IL-12 fusion
20 proteins, except that spleen cells from Balb/c mice activated for 3 days with Concanavalin A, were used instead of PHA-activated human PBMC. A mouse-specific ELISA was used to quantitate the amount of IFN- γ induced by the human p40/mouse p35 hybrid molecules from mouse cells.

For the human system, a quantitative ELISA was developed by coating 96-well plates
25 (Nunc-Immuno plate F96 Cert. Maxisorb) with a mouse monoclonal antibody against human IFN- γ (1 μ g/ml) in phosphate buffered saline (PBS; Pestka Biological Laboratories) overnight at 4°C, washing unbound antibody three times with PBS, and blocking with a solution of 1% bovine serum albumin (BSA) and 1% goat serum in PBS (150 μ l/well for 2 hr at 37°C). After washing the blocked plates four times with PBS, test samples and dilutions of the IFN- γ standard were

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added in a final volume of 100 µl/well. Following an overnight incubation at 4°C, the plates were washed four times with PBS, and a polyclonal rabbit antiserum against human IFN-γ (1/10000 dilution; Petska Biological Laboratories) was added. After an additional incubation for 1 hr at 37°C and four washes with PBS, a polyclonal donkey anti-rabbit detecting antibody, conjugated to horse radish peroxidase (1/700 dilution; Petska Biological Laboratories) was added for 1 hr at 37°C. The plates are then washed four times with PBS and 100 µl of K-blue substrate (ELISA Technologies, Neogen Corp.) was added until the color in the wells containing the standard curve was sufficiently developed, at which time 100 µl of Red-stop solution (ELISA Technologies) was added. The plate was read at 650 nm using an ELISA plate reader (Dynatech MR7000) and the amount of IFN-γ was calculated by comparing the optical density of the test sample with a standard curve derived from the dilutions of the control IFN-γ. The amount of IFN-γ that was induced in the presence of both IL-2 and IL-12 generally ranges from 1200-2000 pg/ml while the amount produced in the absence of IL-12 was generally less than 50 pg/ml.

The biological activity of the culture supernatants described in Example 2 were compared for their ability to stimulate IFN-γ production. As depicted in FIG. 4, the highest activity was obtained with the Fc-p35 fusion protein co-expressed with free p40 subunit, although the other combinations with both subunits were also active. More accurate measurements with purified proteins are described below.

Example 4 Expression of Antibody-IL-12 Fusion Proteins

The experiments described in Example 2 demonstrate that a convenient way to express fusion proteins with the IL-12 heterodimeric cytokine is to co-express a fused p35 subunit protein together with the free p40 subunit in the same cell. This can be done by two approaches: the first is achieved by co-transfecting the fusion protein vector and the p40 expression vector simultaneously (*i.e.*, simultaneous transfection); the second is to first transfect a cell with p40 alone and select for high level, stable secretors of this protein, and then use this cell as a recipient for transfection by the fusion protein expressing construct (*i.e.*, sequential transfection). The latter method is particularly useful when the fusion protein is an antibody molecule with both a heavy and light chain that need to be assembled properly for correct assembly and secretion. Theoretically, the fusion of p35 subunit could be to the heavy or light chain, but the preferred embodiment would be to the carboxyl terminus of the heavy chain, where it can be more free to

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interact with the IL-12 receptor on cells. It is also possible to fuse the p35 subunit via its carboxyl terminus to the amino terminus of the heavy or light chain. In this case, a leader sequence would be required for p35 expression, since it would be at the amino terminus of the fusion protein, thus requiring its direction to the endoplasmic reticulum for assembly and secretion from the cell.

The nucleic acid construct can also include the endogenous promoter and enhancer for the variable region-encoding gene to regulate expression of the chimeric immunoglobulin chain. For example, the variable region encoding genes can be obtained as DNA fragments comprising the leader peptide, the VJ gene [functionally rearranged variable (V) regions with joining (J) segment] for the light chain or VDJ gene for heavy chain, and the endogenous promoter and enhancer for these genes. Alternatively, the gene coding for the variable region can be obtained apart from endogenous regulatory elements and used in an expression vector which provides these elements.

Variable region genes can be obtained by standard DNA cloning procedures from cells that produce the desired antibody. Screening of the genomic library for a specific functionally rearranged variable region can be accomplished with the use of appropriate DNA probes such as DNA segments containing the J region DNA sequence and sequences downstream. Identification and confirmation of correct clones are then achieved by DNA sequencing of the cloned genes and comparison of the sequence to the corresponding sequence of the full length, properly spliced mRNA.

4.1 Simultaneous Transfection

Simultaneous transfection can be achieved by constructing a vector with two transcription units and a selectable marker gene. Such vectors are described for the expression of recombinant antibodies in mammalian cells. Gillies, *et al.*, *J. Immunol. Methods* 125: 191-202 (1989). An alternative method is to use two independent plasmid vectors (one with a transcription unit for the fusion protein and one with a transcription unit for the p40 subunit) with their own selectable marker genes, and to select for successfully transfected, expressing cells by culturing in the presence of the drugs to which the cells have become resistant (*e.g.*, methotrexate in cells transfected with the dhfr gene). Still another approach would be to use an expression vector for the fusion protein to the p35 subunit containing a selectable marker gene and co-transfecting a second vector with no selectable marker gene and a transcription unit for the p40 subunit. Any

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drug resistant clone obtained by the latter method could not secrete the fusion protein in the absence of the p40 subunit and thus, would not be detected by an ELISA assay of the culture supernatant. Only cells transfected with both vectors would secrete the intact fusion protein-p40 heterodimer.

5 A plasmid vector (pdHL7-14.18-p35) was constructed, as described in Gillies, *et al.*, *J. Immunol. Methods* 125: 191-202 (1989), that contains a dhfr-selectable marker gene, a transcription unit encoding a humanized 14.18 anti-GD2 antibody light chain, and a transcription unit encoding a humanized heavy chain fused to the p35 subunit of human IL-12. The fusion was achieved by ligation of the XmaI to XhoI fragment of the adapted p35 subunit cDNA, as
10 described in Example 2, to a unique XmaI site at the end of the CH3 exon of the human IgG1 H chain gene. Both the H and L chain transcription units include a cytomegalovirus (CMV) promoter (in place of the metallothionein promoter in the original reference) at the 5' end and a poly adenylation site at the 3' end. A similar vector (pC-p40) was constructed for expression of the free p40 subunit but did not include a selectable marker gene (dhfr or other) but still used the
15 CMV promoter for transcription. The coding region in this case included the natural leader sequence of the p40 subunit for proper trafficking to the endoplasmic reticulum and assembly with the fusion protein. Another version of this vector (pNC-p40), which includes the neomycin resistance gene, was constructed for use in sequential transfection.

 For simultaneous transfection, plasmid DNAs (approximately 10 µg of each plasmid;
20 pdHL7-14.18-p35 and pC-p40) were linearized by digestion with SalI restriction enzyme, purified using PCR Cleanup kit (Wizard, Promega), and electroporated into 5×10^6 myeloma cells (in 0.5 ml ice cold PBS) using a setting of 0.25 volts and 500 µF. After a recovery for 10 min on ice, cells were transferred to fresh medium and plated in 96-well dishes at approximately 10^5 cells/ml. After 48 hr, cells were fed with medium containing methotrexate (0.1 µM). Fresh medium was
25 added by exchange of half the fluid volume every 4 days until clones appeared. Expression of the desired antibody-IL-12 fusion protein was assayed using an ELISA based on antibody Fc detection. The capture antibody reacted with human H and L chains, and the detection utilized an antibody specific for human Fc. Positive clones were expanded in selection medium and the product was purified by binding to and elution from protein A Sepharose as described above.
30 Eluted proteins were analyzed by PAGE and detected by staining with Coomassie Blue.

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4.2 Sequential Transfection

For sequential transfection, plasmid pNC-p40 was electroporated into cells, as described above, and cells were plated and selected in G418-containing medium. Culture supernatants from drug-resistant clones were tested by ELISA for production of p40 subunit. The capture antibody was a mouse anti-human IL-12 p40 and the detecting antibody was directed to human IL-12 p40/p70. Commercial ELISA kits are available from several manufacturers for this purpose (Pharminogen, San Diego; R & D Systems, MN). The highest producing cell clones were tested for the stable expression of p40. One such clone was transfected with pdHL7-14.18-p35 plasmid DNA, as described above, and clones were selected in methotrexate-containing medium.

Expression of the desired antibody-IL-12 fusion protein was assayed using an ELISA based on antibody Fc detection. The capture antibody reacted with human H and L chains, and the detection utilized an antibody specific for human Fc. Positive clones were expanded in selection medium and the product was purified by binding to and elution from protein A Sepharose as described above. Eluted proteins were analyzed by PAGE and detected by staining with Coomassie Blue.

4.3 Activities of Antibody-IL-12 Fusion Proteins

As summarized in Table 1, fusion protein-expressing cell clones were obtained by either simultaneous transfection and sequential transfection but more highly productive clones were obtained using sequential transfection. The product secreted by two individual transfectants were analyzed for chain composition. The SDS-PAGE analysis is shown in FIG. 5A. Clearly, both clones secrete the same relative amount of each of the three chains: light chain, H chain-p35, and covalently bound p40, indicating complete and proper assembly of this 6-chain molecule. The same process was repeated with a second antibody, KS-1/4, reactive with the EpCAM antigen expressed on virtually all epidermal carcinoma cells (colon, lung, breast, prostate, pancreatic, ovarian, and bladder carcinoma). Exactly the same results were obtained, including normal binding activities of the antibodies to their respective antigens.

The biological activities of the whole antibody-IL-12 fusion proteins are shown in FIG. 5. When assayed for ability to stimulate proliferation of mitogen-activated human PBMC, the Hu-KS-IL-12 fusion protein with both human IL-12 chains was nearly as active on a molar basis as the human IL-12 standard (FIG. 5B). The same construct containing the mouse p35 subunit

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fused to Hu-KS-1/4 was significantly less active in the stimulation of human PBMC. When assayed for ability to induce IFN- γ secretion from PHA-activated PBMC, the Hu-KS-IL-12 protein with human IL-12 chains was about 6-fold less active than the IL-12 standard, while the hybrid form was an additional 4-fold less active (FIG. 5C). When mouse effector cells
 5 (pre-stimulated with Concanavalin A) were used, the hybrid form was about 50-fold less active than the mouse IL-12 standard. The all-human form was inactive (FIG. 5D), as expected from the literature. See, Schoenhaut, *et al.*, *J Immunol.* 148: 3433-3340 (1992).

Table 1
Comparison of Co-transfection and Sequential
Transfection of IL-12 Fusion Protein Expression

<i>Method</i>	<i>Frequency of Positive Clones</i>	<i>Expression Level (ng/ml)</i>
Co-transfection	4/22	20, 22, 244, 386
Sequential	26/37	18, 19, 19, 45, 48, 60, 67, 93, 97, 128, 177, 244, 256, 345, 348, 366, 371, 386, 504, 554, 731, 757, 821, 2000

Example 5 Expression of Single Chain IL-12 Fusion Proteins

10 The methods just described for the production of dimeric antibody and Fc-based fusion proteins can also be used in its simpler form to express single chain fusion proteins with IL-12 (those not forming dimers). In this case, a single polypeptide encoding sequence is joined to the sequence for the p35 subunit and co-expressed in the same cell as the free p40 subunit. Either of the two methods, simultaneous or sequential transfection, can be used to produce single-chain
 15 heterodimeric fusion proteins. The purpose of such fusion proteins can be either to target IL-12 to an antigen bearing cell, through the fusion of a single-chain Fv (sc-Fv) antibody (Huston and Oppermann, WO 88/09344) or to combine the very specific immunostimulatory effect of IL-12 together with a protein antigen as an adjuvant. The linking of stimulatory protein and antigen ensures their co-localization following injection into an animal. The antigen can be any
 20 polypeptide. These can induce antibodies in animals capable of reacting with tumor, viral or other antigens that have therapeutic value. For example, sc-Fv can be used as it is often advantageous

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to induce immune responses to antibody V regions including the idiotype (specific antigen binding region) for the purpose of stimulating idiotype networks.

The type of antigen used for such fusion proteins can also be one that normally induces an allergic response, such as the Der p I and Der p II from dust mites, or tropomyosin from several types of shellfish, which can be fused at the DNA level to the p35 subunit of IL-12 and expressed in the same cell with the p40 subunit. Immunization with such fusion proteins would induce strong Th1 helper cell responses that would be useful in desensitizing the disease-causing Th2 response in atopic patients with allergy.

To demonstrate the expression of a single chain fusion protein, a scFv version of the KS-1/4 antibody was constructed. The 5' end of the protein-encoding portion of fusion gene (an XbaI to AflII fragment) consists of a leader sequence derived from a mouse k light chain, fused to the mature protein sequence of the KS-1/4 L chain V region. The end of the V region is fused, in frame, to a DNA encoding the simple linker sequence, (Gly₄Ser)₃, described by others (Huston and Oppermann, WO 88/09344) followed, in frame, by the sequence encoding the H chain V region of KS-1/4. The 3' end of this scFv contains a XmaI site, compatible with ligation to the 5' end of the human and mouse versions (XmaI to XhoI fragments) of the p35 subunit of IL-12. The final XbaI to XhoI fragments were inserted into the corresponding sites of the same expression vector (pdC) used to express the free IL-12 subunits to give vectors pdC-SCA-hu-p35 and pdC-SCA-mu-p35.

These vectors were introduced into a human p40 expressing cell line and grown in medium containing methotrexate (0.1 μ M). Fusion protein-expressing, drug-resistant clones were identified by ELISA assays specific for the species of p35 utilized in the construct (*i.e.*, an IL-12 human p40 antibody was used for antigen capture, and specific anti-mouse or human-p35 antibodies were used for detection). Culture media from each type of single-chain fusion protein were used to determine their amounts so that relative specific activities could be calculated. Serial dilutions of each sample were tested for the ability to induce IFN- γ secretion as detailed above in Example 2. The results are shown in FIG. 6, which compares the activity of single-chain IL-12 fusion proteins made with either both human subunits or with mouse p35 and human p40, as well as the species specificity of the fusion proteins. The data show that the human IL-12 single chain fusion protein is as active as the whole antibody fusions in its ability to induce IFN- γ

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but that it is not as potent as the human IL-12 standard when human PBMC were used (FIG. 6A). The hybrid mouse/human form was approximately 50-fold less than the mouse IL-12 control as was seen with the whole antibody construct (FIG. 6B). FIG. 6C shows an antigen binding assay of the single-chain IL-12 proteins. Plates were coated with the KS antigen recognized by the
5 KS-1/4 antibody and used to capture any reactive antibody or antibody fusion protein. After washing several times, the bound fusion protein was detected using an anti-human IL-12 p40 antibody. The data show that the single-chain fusion proteins bound to the antigen coated plate and could be detected with an antibody against IL-12, thus demonstrating that the fused molecules retain antigen binding activity. The intensity of binding was roughly 3-fold lower than
10 that seen with the whole KS-1/4 antibody but this is not unexpected, due to the monovalency of the single chain construct.

The activity results with both whole antibody and single chain IL-12 fusion proteins suggest that the amino terminus of the p35 chain may be important to receptor binding since fusions appear to reduce activity. Nonetheless, the antibody-IL-12 molecules are still very potent
15 inducers of IFN- γ at concentrations above 1 ng/ml. The concentration of such molecules in treated animals is expected to be several orders of magnitude higher than this both in the circulation, and at the target site of action.

A possible way to increase the specific activity of antibody-IL-12 fusion proteins would be to insert a flexible peptide linker between the antibody and p35 sequences thus giving more
20 freedom to the amino terminal sequences of this subunit. A sequence such as the (Gly₄Ser)₃ linker, described above, could be used in this manner. One possible problem with this approach is that such a linker could be immunogenic, especially when fused to a powerful immune stimulator such as IL-12.

Example 6 Pharmacokinetic Properties of IL-12 Fusion Proteins

25 The antibody-IL-12 fusion proteins were tested for their pharmacokinetic behavior following intravenous injection into Balb/c mice. Blood was collected from mice by retro-orbital bleeding and stored at 4°C in Eppendorf micro-centrifuge tubes. ELISA methods were used to measure the amount of human antibody, as well as the amount of intact IL-12 fusion protein, remaining in the blood at increasing time points. The first ELISA measuring human antibody
30 utilizes an antibody against human H and L chains for capture and an anti-human Fc antibody for

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detection. The fusion protein-specific assay uses the same first capture step, but an anti-p40 subunit antibody for detection. As depicted in FIG. 7, both the antibody and IL-12 fusion protein had a prolonged half-life but the half-life of the fusion protein was somewhat shorter. This suggests that the circulating fusion protein is cleaved over time to release IL-12 while the antibody remains in the circulation. Earlier-reported experiments with other antibody-cytokine fusion proteins demonstrate that cytokines can be released by protease cleavage. See, Gillies, *et al.*, *Bioconj. Chem.* 4: 230-235 (1993). Nonetheless, the half-lives of the fusion proteins are far longer than the 3 hr value reported for native IL-12. In fact, the serum concentration at 72 hr is still much higher than the level required to induce IFN- γ secretion. Trincieri, *Blood* 84: 4008-4027 (1992).

Example 7 Treatment of established colon carcinoma with antibody-IL-12 fusion protein.

The murine colon carcinoma, CT26, is particularly insensitive to treatment with systemic administration with mouse IL-12 at non-toxic doses. Martinotti, *et al.*, *Eur. J. Immunol.* 25: 137-146 (1995). Some efficacy has been found when systemic IL-12 administration has been combined together with repeated vaccination of irradiated CT26 cells, engineered to secrete IL-2. Vagliani, *et al.*, *Cancer Res.* 56: 467-470 (1996). An alternative approach to successful therapy involved the engineering CT26 to secrete low levels of IL-12. This was ineffective unless mice were first treated with antibodies to deplete CD4⁺ cells, Martinotti, *et al.*, *Eur. J. Immunol.* 25: 137-146 (1995), presumably due to an immunosuppressive effect of these cells after exposure to the engineered tumors *in vivo*. Still another approach of engineering much higher IL-12 secretors was far more successful, thus indicating that the amount of local IL-12 was critical in establishing an immune response to subcutaneous tumors, Colombo, *et al.*, *Cancer Res.* 56: 2531-2534 (1996). In this case, however, there was no demonstration of treatment of established, disseminated tumors similar to what would be seen in the clinical setting. The purpose of the present experiment was to evaluate the efficacy of antibody-IL-12 fusion proteins for the treatment of murine colon carcinoma, CT26.

CT26 cells were transfected with a cDNA encoding the antigen recognized by the KS-1/4 antibody, referred to as either KS antigen (KSA) or epithelial cell adhesion molecule (EpCAM). Clones expressing this protein on their surface were identified by immunostaining with KS-1/4

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and fluorescence activated cell sorting (FACS) analysis. Cells from one clone, stably expressing KSA (clone 21.6), were injected into the tail vein of Balb/c mice (1×10^5 per mouse). Untreated mice formed extensive pulmonary metastases by day 28 and died within 40 days of inoculation. This growth rate was virtually the same as the parental cells indicating that the expression of the human KSA had no effect on CT26 immunogenicity or ability to form tumors.

The efficacy of the antibody-IL-12 fusion protein for therapy of CT26 metastases was tested in this mouse model using the hybrid human/mouse form which has activity on mouse cells. Following tumor cell injection, mice received injections of either PBS (no treatment control), the KS-1/4-IL-2 fusion protein (positive control), KS-1/4 antibody with free IL-2 (negative control) or the KS-1/4-IL-12 fusion protein (test sample). Treatment began on day 4, a time when established metastases are readily detectable by histological staining in the lungs of animals, and continued daily for 5 days. On day 28 after tumor cell inoculation, animals were euthanized and their lungs examined for the presence of tumor. The weights of the lungs were also measured to determine the amount of tumor mass, relative to tumor-free mice. The results are summarized in Table 2. Untreated animals had extensive metastatic disease characterized by near complete surface coverage of the organ with tumor via fusion of individual metastatic nodules. The weights of the lungs increased by an average of three-fold, indicating that the tumor masses actually made up the majority of the organ. Treated animals had little if any evidence of metastases, with some animals completely free of tumor. None of the animals showed any overt sign of toxicity during the treatment process. Thus, unlike treatment with systemic IL-12, antibody-IL-12 fusion protein therapy can eradicate established metastatic CT26 colon carcinoma.

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Table 2

**Treatment of Murine Colon Carcinoma Lung Metastases
in SCID Mice with Antibody-IL-12 Fusion Proteins**

<i>Treatment</i>	<i>Metastatic Score</i>	<i>Organ Weights</i>
PBS	3, 3, 3, 3, 3, 3	0.52
Hu-KS1/4	3, 3, 3, 3, 3	0.48
Hu-KS-1/4 + IL-2	3, 3, 3, 3, 3	0.40
Hu-KS-IL-2	2, 1, 1, 1, 1	0.22
Hu-KS-IL-12	1, 1, 1, 1, 1	0.20

Experimental lung metastases were induced by intravenous injection of 10^5 CT26-KSA cells. Treatment began three days later with intravenous injection of 10 μ g of the humanized KS-1/4 antibody or the indicated fusion protein for five consecutive days.

5 Animals were sacrificed and the metastatic score was determined by the extent of surface coverage: 0= no visible metastatic foci; 1= less than 5% of the surface covered; 2= 5 to 50% of the surface covered; and 3= more than 50% of the lung surface is covered with metastatic foci.

Example 8 IL-12 fusion proteins as vaccines.

10 The humanized KS-1/4 antibody IL-12 fusion protein in PBS buffer, made with the murine p35 subunit (HuKS-1/4-mIL-12), was injected into Balb/c mice intravenously (5 μ g/day x 5). Control mice received the same antibody, in the same amounts, but with no attached IL-12. Neither injection solution contained any other type of adjuvant. On day 10, blood samples were collected into microcentrifuge tubes by retro-orbital bleeding and plasma were prepared by

15 collecting blood samples in plastic tubes containing sodium citrate, followed by centrifugation at full speed in an Eppendorf tabletop microcentrifuge. ELISA plates (96-well) were coated with the HuKS-1/4 protein containing human constant region and used to capture any mouse antibodies made in response to the immunization. After washing away unbound material, the bound mouse antibodies were detected with goat anti-mouse Fc antibody (Jackson

20 ImmunoResearch) coupled to horse-radish peroxidase. Any bound antibodies could be directed to either the human constant regions or the variable region, both of which are shared between the HU-KS-1/4 and the fusion proteins.

As depicted in FIG. 8, there was little or no reactivity to Hu-KS-1/4 without fused IL-12. The fusion protein, on the other hand, induced a strong antibody response in the absence of

25 exogenous adjuvants and despite the fact that the intravenous route of administration is highly

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unfavorable for inducing such responses, compared to either subcutaneous or intraperitoneal administration. Antibodies of the IgG2a isotype, which are typical of IL-12 enhanced responses, were seen in the antibody-IL-12 injected group but not the group injected with the Hu-KS-1/4 antibody.

5 The immunogenicity of IL-12 fusion proteins administered by various routes is tested by injecting a solution of the fusion protein (such as that described above) in PBS or other biocompatible buffer, or a known adjuvant such as Freund's incomplete or complete adjuvant. For example, single or multiple subcutaneous, intradermal or intraperitoneal injections can be given every two weeks. Alternatively, the fusion protein can be administered first by
10 subcutaneous injection and then followed by intraperitoneal injection. Freund's adjuvant cannot be used for human use, due to the irritation at the injection site. Alternative adjuvants such as precipitates of aluminum hydroxide (Alum) are approved for human use and can be used in the present invention. New organic chemical adjuvants based on squalenes and lipids can also be used for injections into the skin.

15

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all
20 changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

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What is claimed is:

- 1 1. A heterodimeric fusion protein comprising a first and a second chimeric chain, said first
2 chimeric chain comprising a portion of an Ig heavy chain linked by a peptide bond to a
3 first subunit of a heterodimeric cytokine, said second chimeric chain comprising a portion
4 of an Ig heavy chain linked by a peptide bond to a second subunit of said heterodimeric
5 cytokine, said first and a second chain being linked by a disulfide bond.
- 1 2. A fusion protein comprising a first chimeric Ig chain comprising a portion of an Ig heavy
2 chain linked by a peptide bond to a first subunit of a heterodimeric cytokine, said first
3 subunit of said cytokine being linked to a second subunit of said cytokine.
- 1 3. The fusion protein of claim 2 further comprising a second chimeric Ig chain comprising a
2 portion of an Ig heavy chain linked by a peptide bond to a first subunit of a heterodimeric
3 cytokine, said first subunit of said cytokine being linked to a second subunit of said
4 cytokine, said first and second chains being linked by a disulfide bond.
- 1 4. A trimeric fusion protein comprising a first and a second chimeric chain linked by a
2 disulfide bond, said first chimeric chain comprising a portion of an Ig heavy chain linked
3 by a peptide bond to a first subunit of a heterodimeric cytokine, said second chimeric
4 chain comprising a portion of an Ig heavy chain linked by a peptide bond to a second
5 subunit of said cytokine, said second subunit being linked by a disulfide bond to another
6 said first subunit of said cytokine.
- 1 5. A fusion protein comprising a chimeric Ig chain comprising a portion of an Ig light chain
2 linked by a peptide bond to a first subunit of a heterodimeric cytokine, said first subunit of
3 said cytokine being linked to a second subunit of said cytokine.
- 1 6. The fusion protein of claim 1, 2, 3, 4 or 5 wherein said fusion protein displays cytokine
2 biological activity.
- 1 7. The fusion protein of claim 1, 2, 3, 4 or 5 wherein said fusion protein displays antigen-
2 binding specificity.
- 1 8. The fusion protein of claim 1, 2, 3, 4 or 5 wherein said fusion protein has a longer
2 circulating half-life than an unlinked heterodimeric cytokine.

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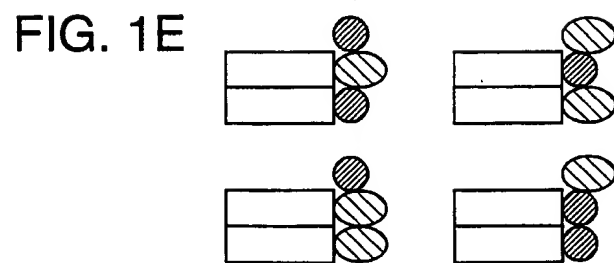
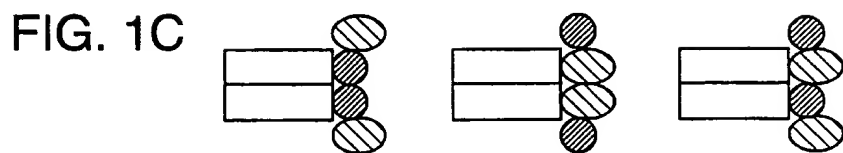
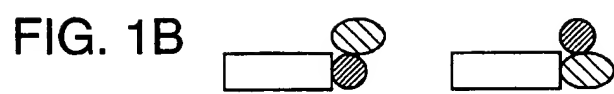
- 1 9. The fusion protein of claim 1, 2, 3 or 4 wherein said portion of an Ig heavy chain further
2 comprises a CH1 domain.
- 1 10. The fusion protein of claim 9 wherein said portion of an Ig heavy chain further comprises
2 a CH2 domain.
- 1 11. The fusion protein of claim 10 wherein said portion of an Ig heavy chain further comprises
2 a CH3 domain.
- 1 12. The fusion protein of claim 1, 2, 3 or 4 wherein said portion of an Ig heavy chain further
2 comprises a CH2 and a CH3 domain.
- 1 13. The fusion protein of claim 1, 2, 3, 4 or 5 wherein said heterodimeric cytokine is IL-12.
- 1 14. A heterodimeric fusion protein comprising a first and a second chimeric chain, said first
2 chimeric chain comprising an antigen linked to a first subunit of a heterodimeric cytokine,
3 said second chimeric chain comprising an antigen linked to a second subunit of said
4 heterodimeric cytokine, said first and a second chain being linked by a disulfide bond.
- 1 15. A fusion protein comprising a first chimeric Ig chain comprising an antigen linked to a first
2 subunit of a heterodimeric cytokine, said first subunit of said cytokine being linked to a
3 second subunit of said cytokine.
- 1 16. The fusion protein of claim 15 further comprising a second chimeric Ig chain comprising
2 an antigen linked to a first subunit of a heterodimeric cytokine, said first subunit of said
3 cytokine being linked to a second subunit of said cytokine, said first and second chains
4 being linked by a disulfide bond.
- 1 17. A trimeric fusion protein comprising a first and a second chimeric chain linked by a
2 disulfide bond, said first chimeric chain comprising an antigen linked to a first subunit of a
3 heterodimeric cytokine, said second chimeric chain comprising an antigen linked to a
4 second subunit of said cytokine, said second subunit being linked by a disulfide bond to
5 another said first subunit of said cytokine.
- 1 18. A method of selectively targeting a heterodimeric cytokine, comprising the step of linking
2 at least one subunit of said heterodimeric cytokine by a peptide bond to a portion of an Ig
3 heavy chain, thereby to form a fusion protein displaying binding specificity for a
4 predetermined antigen and cytokine biological activity.

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- 1 19. A method of selectively targeting a heterodimeric cytokine, comprising the steps of:
- 2 (a) linking a first subunit of said heterodimeric cytokine by a peptide bond to a portion
- 3 of a first Ig heavy chain, thereby forming a first chimeric chain;
- 4 (b) linking a second subunit of said heterodimeric cytokine by a peptide bond to a
- 5 portion of a second Ig heavy chain, thereby forming a second chimeric chain; and
- 6 (c) linking said first and said second chimeric chain by a disulfide bond, thereby
- 7 forming a heterodimeric fusion protein,
- 8 said fusion protein displaying binding specificity for a predetermined antigen and cytokine
- 9 biological activity.
- 1 20. A method of selectively targeting a heterodimeric cytokine, comprising the steps of:
- 2 (a) linking a first subunit of said heterodimeric cytokine by a peptide bond to a
- 3 polypeptide, thereby forming a first chimeric chain, said first subunit of said
- 4 cytokine being linked to a second subunit of said cytokine by a disulfide bond;
- 5 (b) linking a first subunit of said heterodimeric cytokine by a peptide bond to a
- 6 polypeptide, thereby forming a second chimeric chain, said first subunit of said
- 7 cytokine being linked to a second subunit of said cytokine by a disulfide bond; and
- 8 (c) linking said first and said second chimeric chain by a disulfide bond, thereby
- 9 forming a heterodimeric fusion protein,
- 10 said fusion protein displaying binding specificity for a predetermined antigen and cytokine
- 11 biological activity.
- 1 21. A method of increasing the circulating half-life of a heterodimeric cytokine, comprising the
- 2 step of linking at least one subunit of said heterodimeric cytokine to a polypeptide, thereby
- 3 forming a fusion protein having a longer circulating half-life than an unlinked
- 4 heterodimeric cytokine.
- 1 22. The method of claim 21 wherein said polypeptide is selected from the group consisting of
- 2 a portion of an Ig heavy chain, a portion of an Ig light chain, an antigen, and serum
- 3 albumin.

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- 1 23. A method of increasing the circulating half-life a heterodimeric cytokine, comprising the
2 steps of:
- 3 (a) linking a first subunit of said heterodimeric cytokine by a peptide bond to a
4 polypeptide, thereby forming a first chimeric chain, said first subunit of said
5 cytokine being linked to a second subunit of said cytokine by a disulfide bond;
- 6 (b) linking a first subunit of said heterodimeric cytokine by a peptide bond to a
7 polypeptide, thereby forming a second chimeric chain, said first subunit of said
8 cytokine being linked to a second subunit of said cytokine by a disulfide bond; and
- 9 (c) linking said first and said second chimeric chain by a disulfide bond, thereby
10 forming a heterodimeric fusion protein,
- 11 said fusion protein having a longer circulating half-life than unlinked first and second
12 heterodimeric cytokines.
- 1 24. The method of claim 23 wherein said first and second polypeptides are selected from the
2 group consisting of a portion of an Ig heavy chain, a portion of an Ig light chain, an
3 antigen, and serum albumin.



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1 2 3 4 5 6



Fc-p40
Fc-p35
p40
p35

FIG. 2

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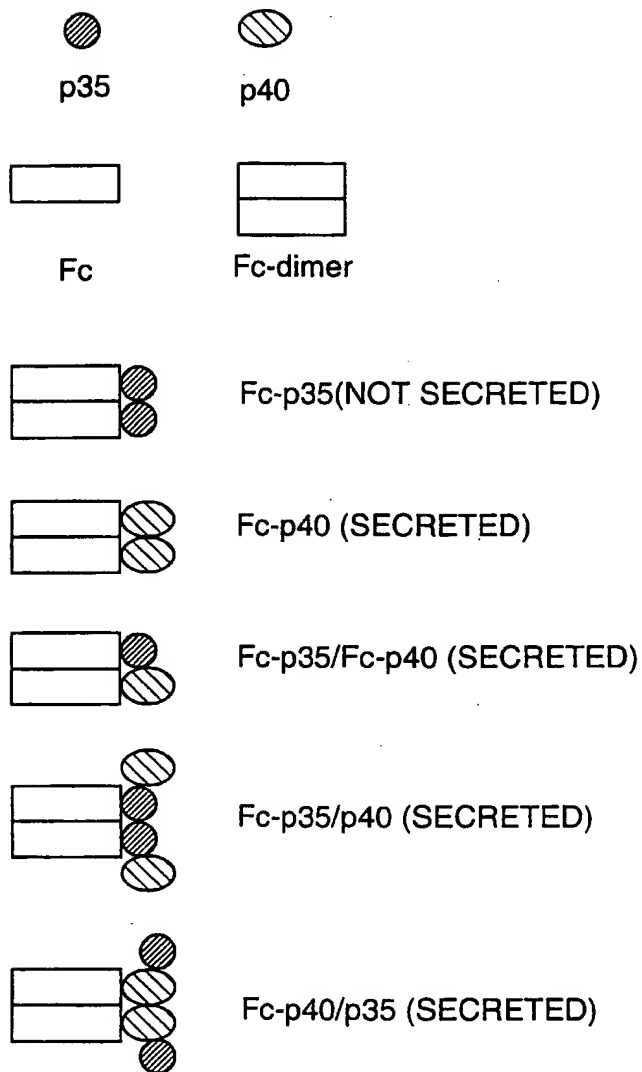
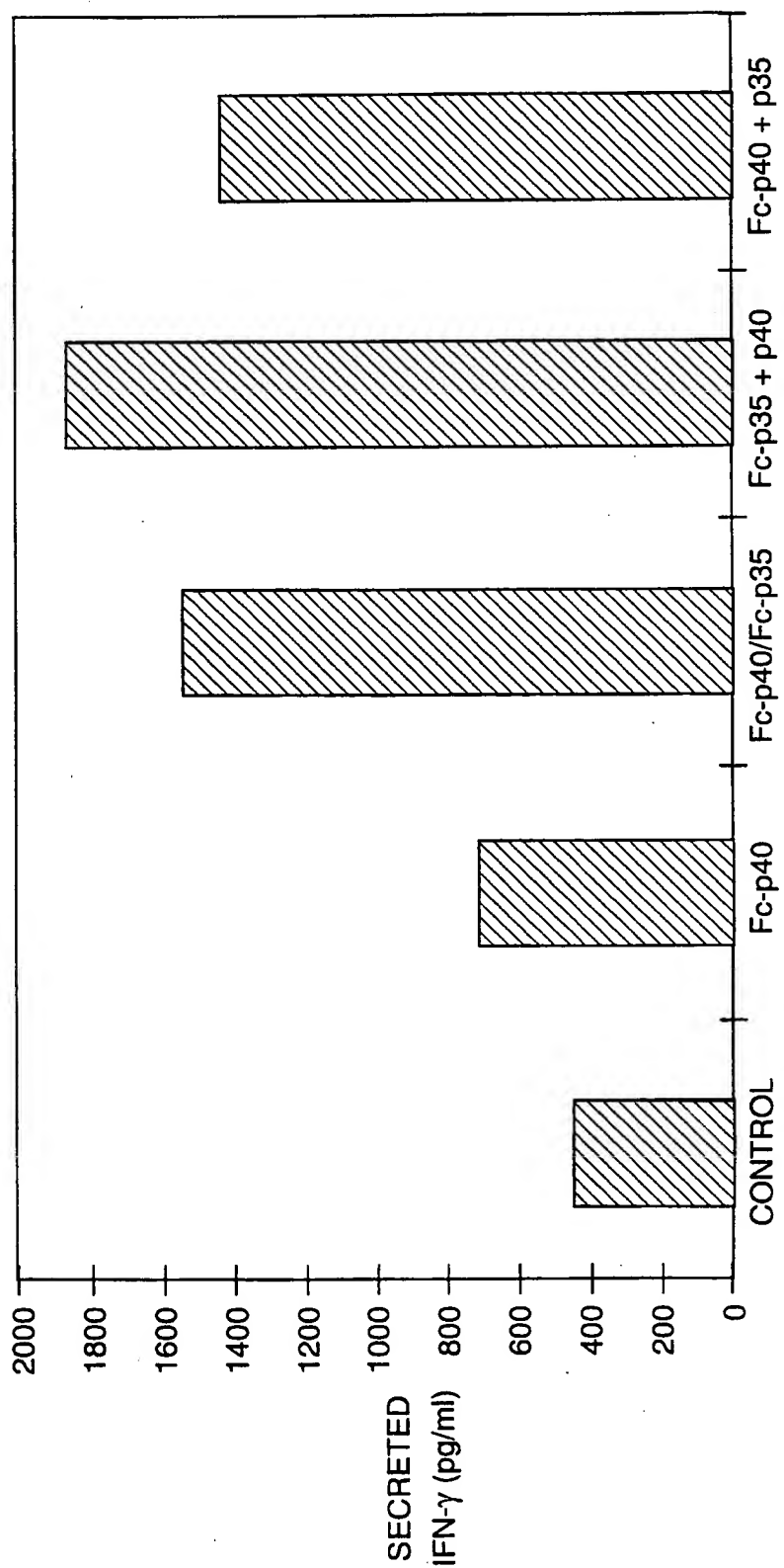


FIG. 3

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CONSTRUCT TESTED

FIG. 4

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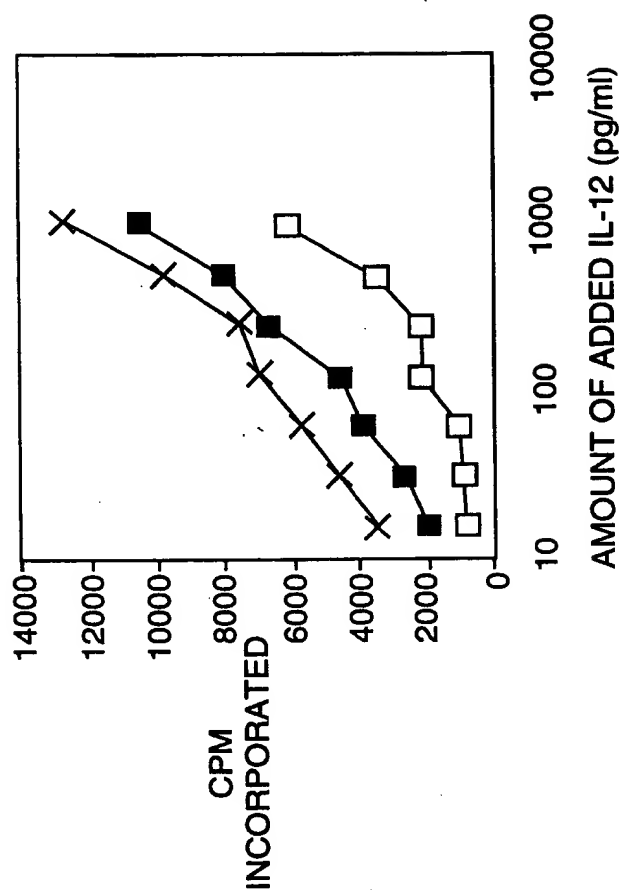


FIG. 5B

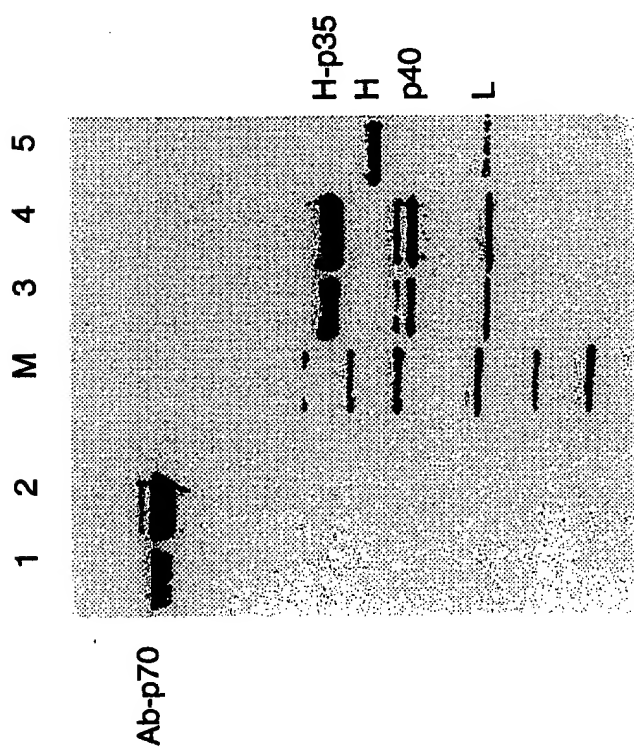


FIG. 5A

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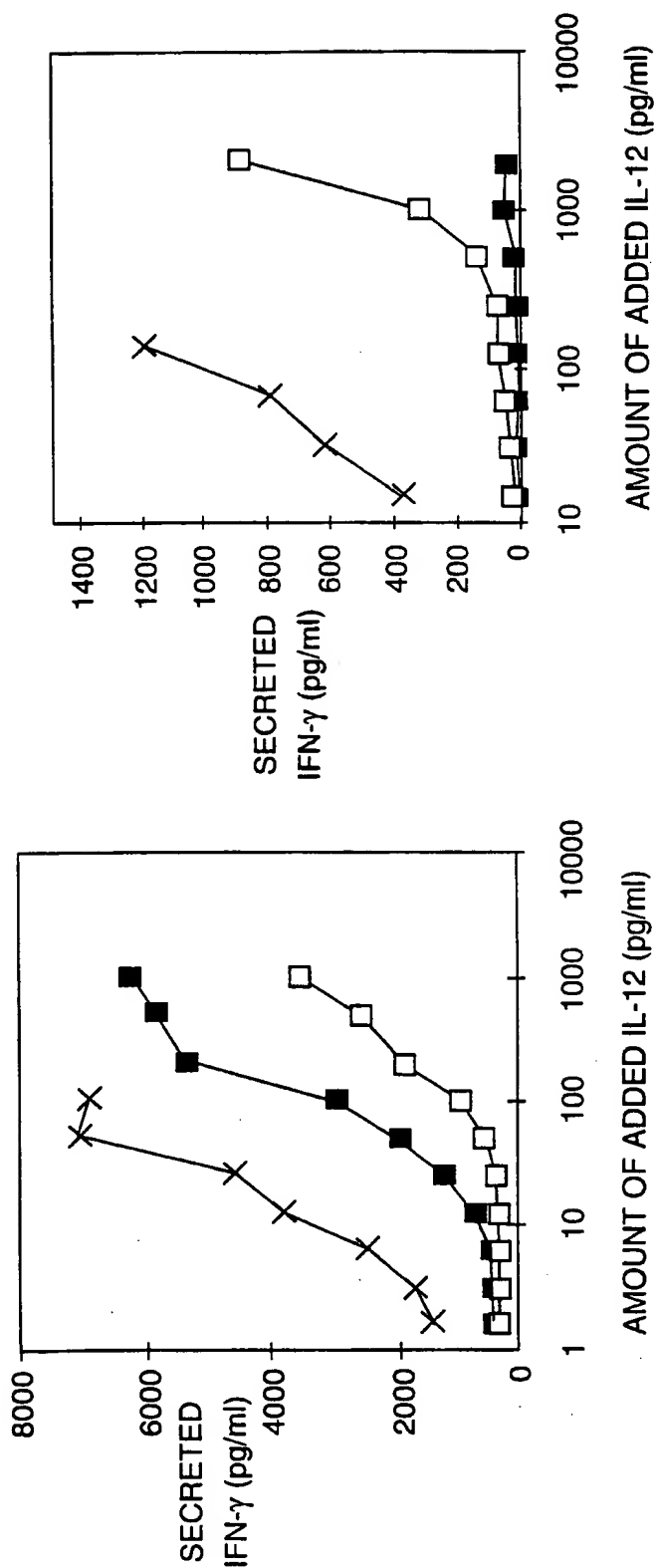


FIG. 5D

FIG. 5C

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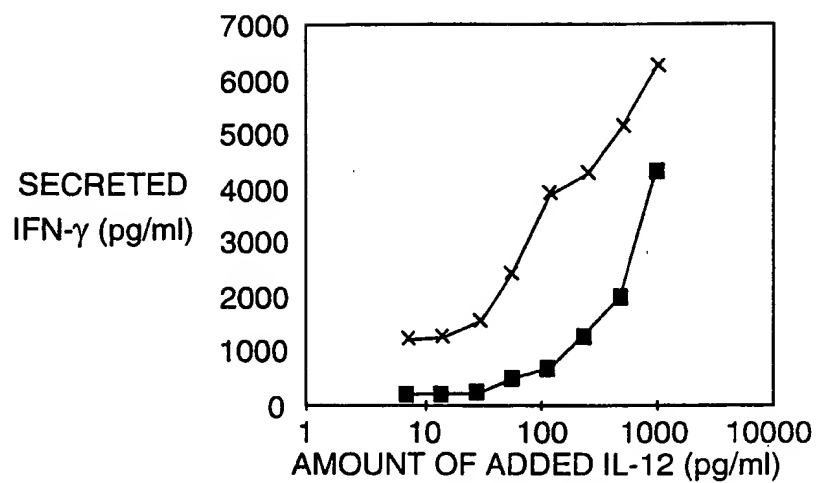


FIG. 6A

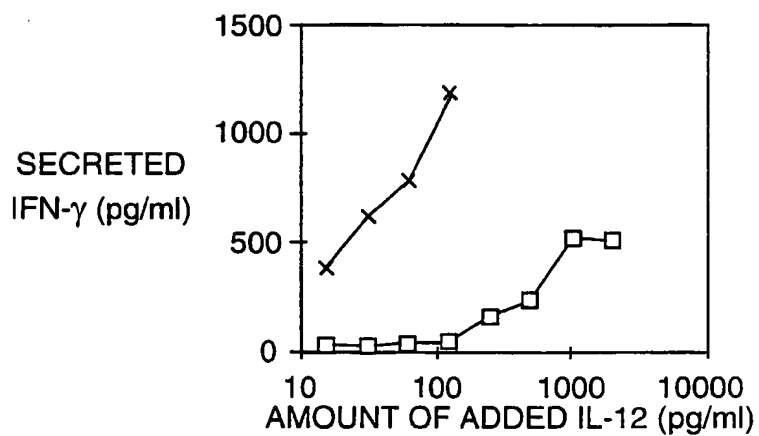


FIG. 6B

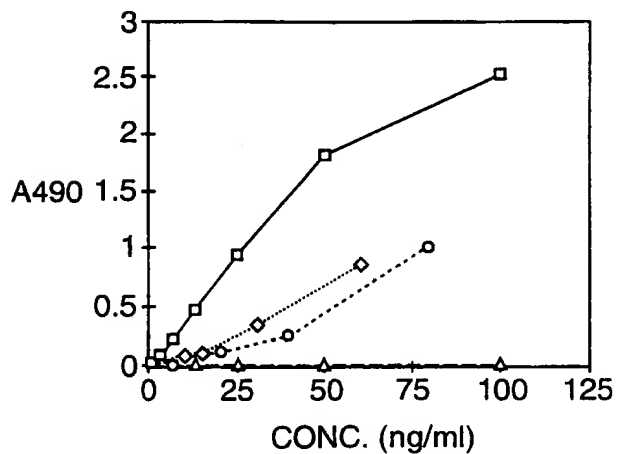


FIG. 6C

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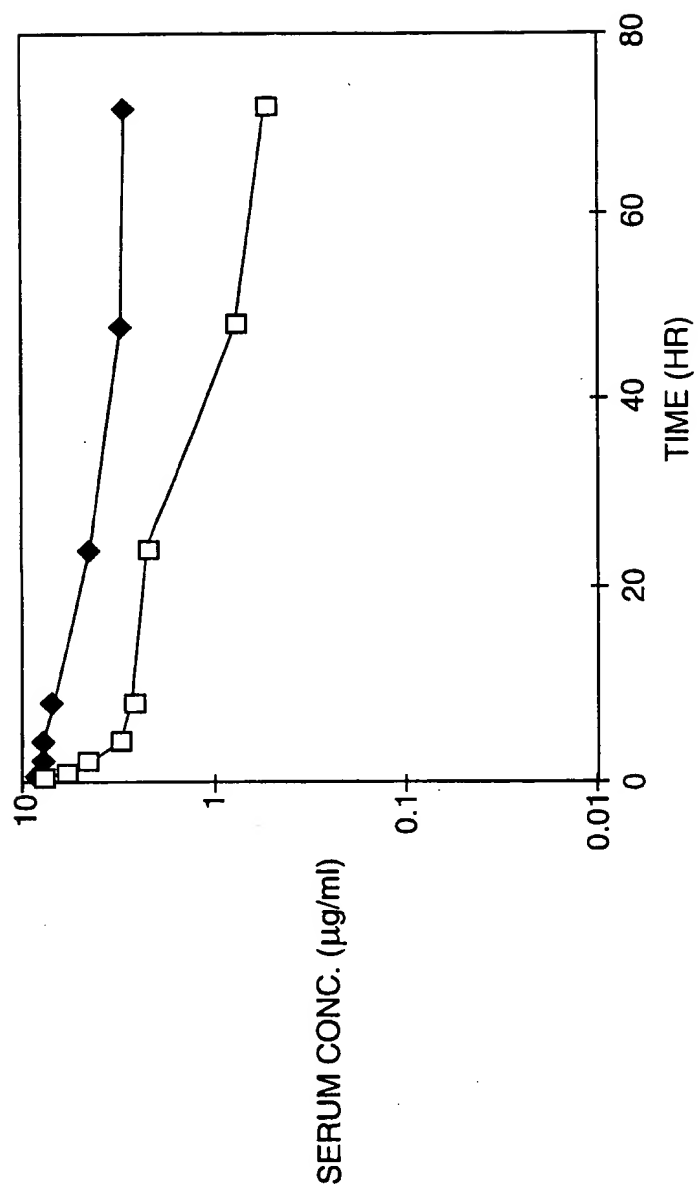


FIG. 7

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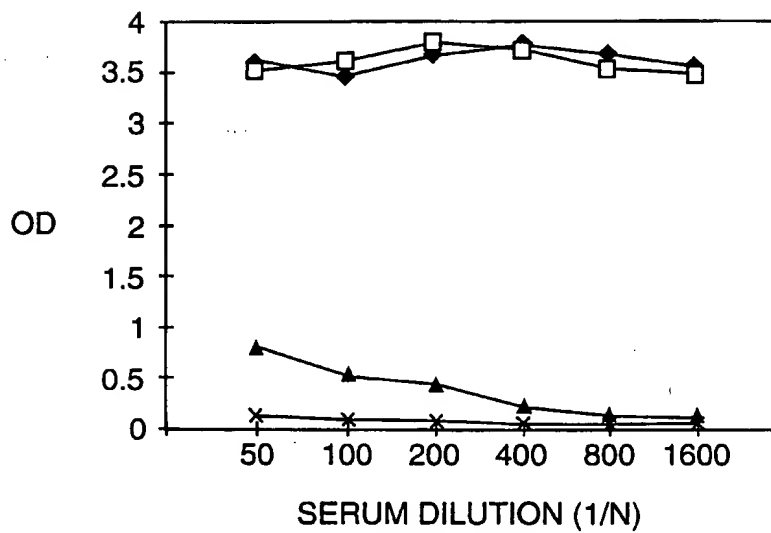


FIG. 8A

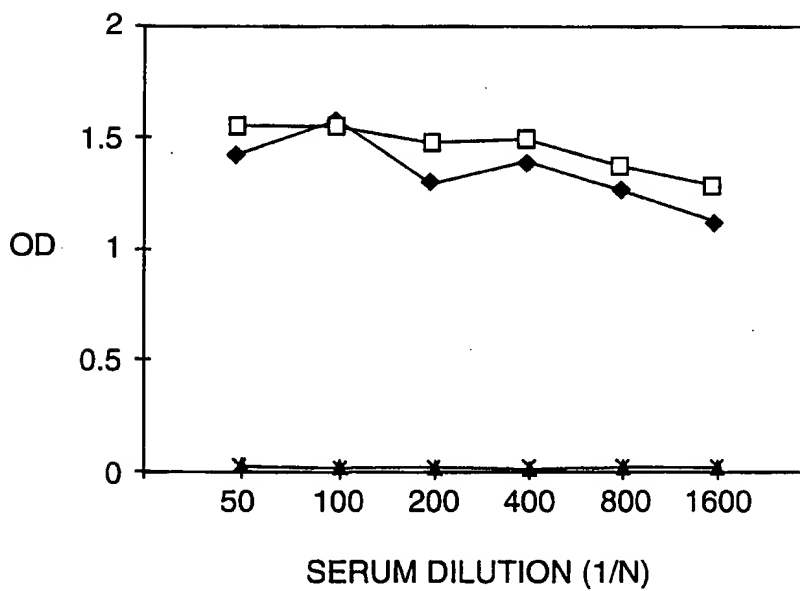


FIG. 8B

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SEQUENCE LISTING

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LAN, Yan
LO, Kin-Ming
LEXIGEN PHARMACEUTICALS CORPORATION

<120> Heterodimeric Fusion Proteins Useful for Targeted
Immune Therapy and General Immune Stimulation

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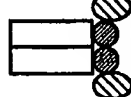








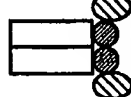








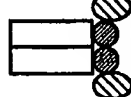

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18



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 19/00, A61K 38/20, 47/48 // C07K 14/54, 16/30	A3	(11) International Publication Number: WO 99/29732 (43) International Publication Date: 17 June 1999 (17.06.99)														
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> (21) International Application Number: PCT/US98/25978 (22) International Filing Date: 8 December 1998 (08.12.98) (30) Priority Data: 08/986,997 8 December 1997 (08.12.97) US (71) Applicant: LEXIGEN PHARMACEUTICALS CORPORATION [US/US]; 125 Hartwell Avenue, Lexington, MA 02173 (US). (72) Inventors: GILLIES, Stephen, D.; 159 Sunset Road, Carlisle, MA 01741 (US). LO, Kin-Ming; 6 Carol Lane, Lexington, MA 02173 (US). LAN, Yan; 21 Newton Street, Belmont, MA 02178 (US). (74) Agent: WALLER, Patrick, R., H.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US). </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 26 August 1999 (26.08.99) </td> </tr> </table>			(21) International Application Number: PCT/US98/25978 (22) International Filing Date: 8 December 1998 (08.12.98) (30) Priority Data: 08/986,997 8 December 1997 (08.12.97) US (71) Applicant: LEXIGEN PHARMACEUTICALS CORPORATION [US/US]; 125 Hartwell Avenue, Lexington, MA 02173 (US). (72) Inventors: GILLIES, Stephen, D.; 159 Sunset Road, Carlisle, MA 01741 (US). LO, Kin-Ming; 6 Carol Lane, Lexington, MA 02173 (US). LAN, Yan; 21 Newton Street, Belmont, MA 02178 (US). (74) Agent: WALLER, Patrick, R., H.; Testa, Hurwitz & Thibault, LLP, High Street Tower, 125 High Street, Boston, MA 02110 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 26 August 1999 (26.08.99)												
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(54) Title: HETERODIMERIC FUSION PROTEINS USEFUL FOR TARGETED IMMUNE THERAPY AND GENERAL IMMUNE STIMULATION																
(57) Abstract <p>Disclosed are methods for producing fusion proteins with the heterodimeric cytokine, interleukin-12. In order to insure that the proper ration of fused and non-fused subunits are obtained in the fusion protein, a specific stepwise approach to genetic engineering is used. This consists of first expressing the non-fused p40 IL-12 subunit in a production cell line, followed by or simultaneously expressing in the same cell, a second recombinant fusion protein consisting of the fused polypeptide linked by a peptide bond to the p35 subunit of IL-12. Molecules containing the p35 fusion protein cannot be secreted from the transfected mammalian cell without first complexing in a one to ratio with the p40 subunit, thus ensuring the production of active heterodimeric fusion proteins.</p>																
<table style="width: 100%; border: none;"> <tr> <td style="text-align: center; width: 50%;">  p35 </td> <td style="text-align: center; width: 50%;">  p40 </td> </tr> <tr> <td style="text-align: center;">  Fc </td> <td style="text-align: center;">  Fc-dimer </td> </tr> <tr> <td style="text-align: center;">  </td> <td style="text-align: center;">Fc-p35(NOT SECRETED)</td> </tr> <tr> <td style="text-align: center;">  </td> <td style="text-align: center;">Fc-p40 (SECRETED)</td> </tr> <tr> <td style="text-align: center;">  </td> <td style="text-align: center;">Fc-p35/Fc-p40 (SECRETED)</td> </tr> <tr> <td style="text-align: center;">  </td> <td style="text-align: center;">Fc-p35/p40 (SECRETED)</td> </tr> <tr> <td style="text-align: center;">  </td> <td style="text-align: center;">Fc-p40/p35 (SECRETED)</td> </tr> </table>			 p35	 p40	 Fc	 Fc-dimer		Fc-p35(NOT SECRETED)		Fc-p40 (SECRETED)		Fc-p35/Fc-p40 (SECRETED)		Fc-p35/p40 (SECRETED)		Fc-p40/p35 (SECRETED)
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/25978

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K19/00 A61K38/20 A61K47/48 //C07K14/54,C07K16/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>T. KIM ET AL.: "An ovalbumin-IL-12 fusion protein is more effective than ovalbumin plus free recombinant IL-12 in inducing a T helper cell type 1-dominated immune response and inhibiting antigen-specific IgE production."</p> <p>THE JOURNAL OF IMMUNOLOGY, vol. 158, no. 9, 1 May 1997, pages 4137-4144, XP002106573 Baltimore, MD, USA see abstract see figure 1 see page 4143, left-hand column, line 7 - line 12</p> <p style="text-align: center;">--- -/--</p>	<p>2,5-13, 15,18, 21,22</p>



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Patent family members are listed in annex.

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Date of the actual completion of the international search

18 June 1999

Date of mailing of the international search report

06/07/1999

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NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/25978

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 05468 A (LYNXVALE LTD.) 23 February 1995 see claims ---	1,6-13, 18,19, 21,22
X	WO 96 18412 A (BETH ISRAEL HOSPITAL ASSOCIATION) 20 June 1996 see page 9, line 4 - line 26 see claims ---	1,6-13, 18,19, 21,22
A	G. LIESCHKE ET AL.: "Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity in vivo." NATURE BIOTECHNOLOGY, vol. 15, no. 1, January 1997, pages 35-40, XP002106574 New York, NY, USA see abstract ---	1-24
A	M. MARK ET AL.: "Expression and characterization of hepatocyte growth factor receptor-IgG fusion proteins." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 36, 25 December 1992, pages 26166-26171, XP002106575 Baltimore, MD, USA see abstract ---	1-24
A	WO 92 08495 A (ABBOTT BIOTECH, INC.) 29 May 1992 see examples see claims ---	1-24
A	WO 97 20062 A (UNIVERSITY OF MASSACHUSETTS & BETH ISRAEL HOSPITAL) 5 June 1997 see the whole document ---	1-24
P,X	S. GILLIES ET AL.: "Antibody-IL-12 fusion proteins are effective in SCID mouse models of prostate and colon carcinoma metastases." THE JOURNAL OF IMMUNOLOGY, vol. 160, no. 12, 15 June 1998, pages 6195-6203, XP002106576 Baltimore, MD, USA see the whole document -----	1-24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/ 25978

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 18-20 (all partially, as far as an in vivo method is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/25978

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9505468	A	23-02-1995	NONE	
WO 9618412	A	20-06-1996	EP 0793504 A JP 11501506 T	10-09-1997 09-02-1999
WO 9208495	A	29-05-1992	AU 660297 B AU 9059691 A CA 2095836 A EP 0574395 A JP 9506761 T US 5650150 A	22-06-1995 11-06-1992 10-05-1992 22-12-1993 08-07-1997 22-07-1997
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